Original Paper

DOI: 10.22067/veterinary.v8i1.51335

Received: 11 November, 2015
Accepted after revision: 29 November, 2016
Published online: 10 April, 2017

Detection of *Giardia duodenalis* antigen in companion rabbits of Ahvaz district, South-West of Iran

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Keywords

Giardia duodenalis, prevalence, rabbit, Ahvaz

Abstract

Giardia duodenalis is an important zoonotic protozoon, found in the small intestine of humans and mammals. There is very little information available regarding rabbits infected with Giardia in Iran. The objective of the present study is to detect *Giardia duodenalis* antigen in companion rabbits of the Ahvaz district, South-West of Iran. A total of 58 fecal samples of companion rabbits that had been collected during January 2011 to December 2014 were submitted to the parasitology laboratory of the Veterinary Faculty for *Giardia duodenalis* testing by two techniques: centrifugation-flotation and a commercial Giardia Antigen Test Kit (BVT Co., Ltd, Lion, France) by immunochromatography assay (ICA). The studied rabbits were categorized into two age groups (less than 1 year old and above or equal to 1 year old), four season. In addition, they were categorized into two other groups based on the stool sample status (diarrheic and non-diarrheic). Five out of fifty eight fecal samples (8.62%) were positive for antigen of *Giardia duodenalis* by ICA. Prevalence was significantly higher in diarrheic rabbits (45.45%; 5 out of 11) than non-diarrheic rabbits (0%; 0 out of 47) (P=0.002). The infection had more prevalence in rabbits older than 1 year (9.09%; 3 out of 33) compared with young rabbits that were less than 1 year old (8%; 2 out of 25). Nevertheless, the difference was not significant. Prevalence was higher in male rabbits (9.52%; 2 out of 21) compared with female rabbits (8.11%; 3 out of 37) and in the season of autumn (15.38%; 2 out of 13), but without a significant difference between the prevalence of infection relative to host gender and season. Microscopy examination on fecal samples showed that 5.17% (3 out of 58) were positive. For greater certainty, three stool samples were collected from each animal at 48 hour intervals. The results indicated that the Giardia antigen was present as a zoonotic disease in rabbits of the Ahvaz district. The obtained data showed that more sensitive techniques such as ICA may be necessary and yield more reliable results in the detection of low levels of Giardia in fecal samples.

Abbreviations

G.: Giardia
G. duodenalis: *Giardia duodenalis*
ICA: Immunochromatography Assay

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

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Introduction

Giardia duodenalis (syn. *G. intestinalis, G. lamblia*) is a protozoan parasite which is found in the small intestine of vertebrates including humans and mammals (Thompson et al., 2000; Barr, 2006). Molecular and phylogenetic analyses of G duodenalis isolates has identified eight distinct genetic groups (assemblages A–H), which differ in their host distribution (Monis et al., 2009; Takumi et al., 2012; Jenkins et al., 2012). Assemblages A and B are associated with human infection, but they are also found in many other mammals (Sprong et al., 2009). Recent molecular findings have shown the presence of potentially *G. duodenalis* assemblages (mainly B) in rabbits (Lebbad et al., 2010; Levecke et al., 2011; Karim et al., 2015). Giardia is responsible for gastroenteritis and diarrhea, depending on the age and health of the infected host as well as the genetic background of the parasite. Transmission is fecal-oral route by ingestion of feces or fecal-contaminated water, food or fomites. There are two stages to the life cycle. Trophozoites are the active motile form. They move towards the colon, where they produce a cyst wall. The cysts are extremely hard and can survive for long periods in water. The parasite has a one to two week incubation period (Adam, 2001; Barr, 2006).

The diagnosis of Giardia infection traditionally has depended on microscopic identification of trophozoites or cysts in feces of the affected animals. Although flotation is a standard method for the detection of Giardia cysts, it is suggested that an alternative test is also needed because microscopic examination is time consuming and needs an experienced microscopist (Barr, 2006). For reliable diagnosis of the intestinal parasites, several laboratory methods have been developed to detect antigens in the fecal samples, such as PCR, ELISA, direct and indirect immunofluorescence assay, monoclonal antibodies or other molecular techniques. Although these tests are more sensitive, specific and more reproducible, they can be expensive and generally take time to be analyzed in a specialized laboratory (Dryden et al., 2006). Recently, a commercial Giardia Antigen Test Kit (BVT Co., Ltd, Lion) by immunochromatography assay (ICA), was released for detection of *G. duodenalis* antigen in animal feces. This test is a rapid enzyme immunoassay that can be conducted on fresh feces and previously frozen feces. Sensitivity and specificity for kits of Giardia Ag Test were found to be nearly 95.6% and 100%, respectively (Geurden et al., 2008). No study has been reported on the distribution of Giardiasis in a population of rabbits in Iran. We conducted this study in order to determine the prevalence of *Giardia duodenalis* infection in the companion rabbits in the Ahvaz area, southwestern Iran. The results of this study can be important for small animal veterinarians.

Materials and Methods

Study area and sample population

In the present study, a total of 58 companion rabbits of different ages were examined for antigen of *Giardia duodenalis* in their fecal samples by immunochromatography assay and for detection of cyst or trophozoite by microscopic examination (flotation method). The rabbits used in this survey, were referred cases to the Veterinary Hospital of the Shahid Chamran University of Ahvaz from January 2011 to December 2014. Ahvaz is located at an elevation of 12 meters above sea level and the climate is warm-humid. Most of the rabbits had been referred for other reasons - mostly for checkup. The samples were stored in an ice chest and transported to the laboratory to be processed. For greater certainty and because of the intermittent nature of *Giardia* shedding, three stool samples were collected from each animal at 48 hour intervals, producing a total of 174 stool samples. Information about the studied rabbits was taken from their owners (exact age, onset of signs, duration of illness and so on). They were divided into two groups (diarrheic and non-diarrheic) based on the stool sample status and into two age groups (less than 1 year old and above or equal to 1 year old). Classification was made by sex and season too. Most of the studied rabbits were laboratory white. Their age was estimated by dental formulary and owner's information.

Laboratory methods

Two methods were employed in order to detect *Giardia*: Fecal centrifugation-flotation technique and immunochromatography assay (ICA).

Fecal centrifugation-flotation technique

Fecal samples (1 g) of 58 rabbits (×3) were examined microscopically for the presence of *G. duodenalis* cysts by flotation in 33% zinc sulphate solution (specific density 1.27). It was filtered through gauze, and centrifuged in a 15 ml tube at 400 g for 10 min. A drop of the float from the meniscus was examined microscopically at 400x magnification for the presence of *G. duodenalis* cyst (Dryden et al., 2006). Trophozoites will not be detected by flotation techniques because the flotation solution lyses the trophozoites. Therefore, direct fecal smears were carried out for demonstration of trophozoites.

Immunochromatography assay and interpretation of the test

Fecal samples were collected from the companion rabbits using the sample collection. We added a volume of 1 full spoon of fecal sample into the buffer diluent. Then the vial was closed and shaken for homogenization. We placed labels on the sample tube for identification and took out the sample spoon provided with the kit. The strips were left in the solution for one minute. Then they were removed and placed on a flat and horizontal surface for migration. Rapid detection of soluble *Giardia duodenalis* cyst antigens



Figure 1: Positive sample of rapid *Giardia* Ag test in companion rabbits in Ahvaz district, Iran by ICA, 2011-2014.



Figure 2: Negative sample of rapid *Giardia* Ag test in companion rabbits in Ahvaz district, Iran by ICA, 2011-2014.

(BVT Co., Ltd, Lion) is a qualitative test. A positive result indicates the presence of Giardia cysts in the feces. One blue and one colored line are positive (Figure 1). One blue colored line is negative (Figure 2). Speed Giardia helps us detect the presence of cyst for a concentration higher than 80 cysts per gram of feces (Geurden et al., 2008).

Statistical analysis

Rabbits were grouped based on age, sex, season and the stool sample status (diarrheic and non-diarrheic), to determine whether these factors were associated with *G. duodenalis* infection, using Chi-square analysis, Fisher's exact test and Z test. Statistical comparisons were carried out using SPSS 16.0 statistical software. Differences were considered significant when $p< 0.05$.

Results

Five out of fifty eight fecal samples (8.62%) were positive for antigen of *Giardia duodenalis* by ICA. Prevalence was significantly higher in diarrheic rabbits (45.45%; 5 out of 11) when compared with non-diarrheic rabbits (0%; 0 out of 47) (P=0.002). The infection had more prevalence in rabbits older than 1 year (9.09%; 3 out of 33) compared with young rabbits who were younger than 1 year (8%; 2 out of 25). Nevertheless, the difference was not significant. Prevalence was higher in male rabbits (9.52%; 2 out of 21) compared with female rabbits (8.11%; 3 out of 37) and in the season of autumn (15.38%; 2 out of 13), but without a significant difference between the prevalence of infection relative to host gender and season. Microscopy examination of fecal samples showed that 5.17% (3 out of 58) were positive. All the rabbits were improved with the administration of metronidazole and other supportive treatments. Prevalence in other seasons (winter, summer and spring) were (9.09%; 1 out of 11, 6.25%; 1 out of 16, and 5.56%; 1 out of 18) respectively. All of the affected rabbits had access to open environment. The results are summarized in Tables 1 and 2:

Discussion

The present survey that is the first report on the prevalence of *Giardia duodenalis* in companion rabbits in Iran; revealed that the overall prevalence of the infection was 8.62 and 5.17% by using immunochromatography assay and fecal centrifugation-flotation technique, respectively. Among zoonotic parasites, the flagellate *G. duodenalis* is known to cause gastroenteritis in a wide range of vertebrates including small mammals (Pantchev et al., 2005). The results highlight the potential role of rabbits for zoonotic transmission of Giardia.

Yet to date, few epidemiological and molecular studies have been conducted on these companion animals. In a recent study from China, it was shown that Giardia cysts were detected in 7.4% (28 out of 378) tested samples of rabbits there. This study also showed that diarrheic animals were more at risk of being infected by Giardia (Zhang et al., 2012). A similar rate of infection (7.6%; 40 out of 528) was found in a recent report from Germany in which ELISA kits were used for diagnosis (Pantchev et al., 2014).

In the present study, among the various methods used, higher infection rate was detected by ICA than microcopy examination. These results show that ICA is more sensitive and reliable than centrifugation–flotation. For these reasons, feces of the rabbits were examined by the above

Table 1
Prevalence of *Giardia duodenalis* infection in companion rabbits of different age and sex in Ahvaz district, Iran by ICA, 2011-2014.

	Age < 1 year		Age ≥ 1 year	
	Neg.	**Pos.**	**Neg.**	**Pos.**
Male	12	1	7	1
Female	11	1	23	2
Total = 58	23	2	30	3

Table 2
Prevalence of *Giardia duodenalis* infection in companion rabbits of different age and season in Ahvaz district, Iran by ICA, 2011-2014.

	Age < 1 year		Age ≥ 1 year	
	Neg.	Pos.	Neg.	Pos.
Summer	6	0	9	1
Winter	7	0	3	1
Spring	4	1	13	0
Autumn	6	1	5	1
Total=58	23	2	30	3

two techniques. It is shown that the estimated amount of Giardia antigens can widely vary in positive stool samples, for instance above 80 cyst in 1 gram feces is found positive to *Giardia* by ICA. Rapid *Giardia* test kit appears to be sufficiently sensitive to detect cases of infection when fairly high levels of antigens are shed (Geurden et al., 2008). More levels of ICA positives and lack of confirmation by microscopy may be due to the low numbers of cysts in fecal samples and microscopy was not sensitive enough to detect these low levels. Trophozoites rapidly disintegrate in feces over time, thus reducing the likelihood of microscopic identification (Barr, 2006). In our survey, there were two samples that were positive on the ICA test, but negative by microscopy examination. In a study in humans, the infection rate was 13.3% by microscopy and 16.7% by ELISA (Sadaka et al., 2015). These results confirm our findings.

Diarrhea has been reported as the major clinical disease for Giardia infection. However, subclinical infection is likely to be also common (Read et al., 2002; Barr, 2006). Interestingly, diarrhea was an important sign of infection in the present study, because the prevalence of infection was significantly more common in diarrheic rabbits in comparison to non-diarrheic rabbits (P=0.002). These data show that diarrhea is a risk factor for individual pathogens in rabbits.

It is likely that the outcome of infection is a complex phenotype and that host factors and co-infections (other parasites or bacteria) will also affect the development of the disease (Monis et al., 2009). Immaturity is considered a significant risk factor for infection in humans and dogs, suggesting that immunity develops with age. Animal behavior, particularly the habit of biting and licking objects which can be contaminated with Giardia cysts, may also be a significant contributing factor (Barr, 2006).

In the present survey, the infection had more prevalence in rabbits above or equal to 1 year in age compared with young rabbits that were less than 1 year old. Perhaps the reason is that the amount of exposure increases with age. Nevertheless, the difference was not significant. In our study, all of the affected rabbits had access to open environment. Surveys in many countries have shown that infection with Giardia is relatively common and widespread in crowded and open environments. Higher prevalence in dense populations may be expected because of the increased ease of transmission (Barr, 2006). The higher prevalence was seen in male rabbits than females in the present study. These results can be explained by the behavioral differences, as males have wider areas of operation than females. Of course, the difference was not significant between the two sexes. As a general rule, it does not seem that sex is a determining factor of infection as other studies have concluded (Epe et al., 2004). Regarding seasonal variation in the prevalence of Giardia, seasonal effects on the infection rate may reflect climatic changes on the parasite, host physiology or in the photoperiod (Barr, 2006). In the present study, the difference was no significant between the four seasons.

Rabbits, despite showing a lower prevalence, may also pose a zoonotic risk based on sequence identities and coincidence of infection in the same household (Lebbad et al., 2010; Soliman et al., 2011). Interestingly, co-infections or repeated infections are considered to be predisposing factors of disease in rabbits, where the outcome of experimental infections ranges from asymptomatic to severe diseases with anorexia, soft discolored feces, watery diarrhea, weight loss and even death (Pantchev et al., 2014). This variability in prevalence rate may be due to geographical variation or difference in the number of animals and type of population surveyed. It may also be attributed to different sensitivity of the diagnostic procedure, difference in sample preservation methods and the criteria used for sample inclusion (Barr, 2006).

Excretion of *G. duodenalis* cysts is intermittent in symptomatic and asymptomatic animals (Barr, 2014). Therefore, just one negative fecal exam may not necessarily mean that the animal is not parasitized by these protozo-

ans. Diagnosis can be improved by repeating the examinations whenever possible. It was found that examination of three samples from the same animal increased the likelihood of positive results (Mundim et al., 2007). In the present study, three samples were collected from each rabbit at 48 hour intervals for greater certainty due to intermittent excretion of trophozoites and cysts. The methods used for detection of parasites are likely to play a great role in the variable prevalence detected worldwide.

In conclusion, this is the first research on the prevalence of *Giardia duodenalis* infection in companion rabbits with alimentary signs. Due to close contact of rabbits with humans and the fact that children play outdoors on the soil, they can be an important potential route of transmission of zoonotic parasite such as *Giardia*. Small mammals have an important role in contamination of the environment to cyst or trophozoite. Our results indicate that the parasite antigen is present in the Ahvaz district, Southwest of Iran. It suggests that climatic conditions in this area (warm and humid) are relatively suitable for the spread and survival of the cysts. It is possible that companion rabbits can be a potential source of environmental contamination in this area. Prevalence data are an essential component for evaluation of zoonotic risk (Barr, 2006). Our results will be the basis of further studies that will permit to deepen our knowledge of the epidemiology of *G. duodenalis*. Based on the fact that this parasite belongs to zoonotic pathogens, more extensive studies are needed in rabbits in different areas to better characterize the transmission and to assess the public health significance of this pathogen.

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Abstract (in Persian)

DOI: 10.22067/veterinary.v8i1.51335

تعیین آنتی ژن *ژیاردیا دئودنالیس* در خرگوش‌های خانگی شهرستان اهواز، جنوب غرب ایران

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دریافت مقاله : ۱۳۹۵/۰۸/۲۰　　　*پذیرش نهایی:۱۳۹۵/۰۹/۰۹*

چکیده

ژیاردیا دئودنالیس، یک تک‌یاخته مهم مشترک بین انسان و حیوان بوده و در روده باریک انسان و پستانداران یافت می‌شود. اطلاعات بسیار کمی، در ارتباط با خرگوش‌های آلوده به ژیاردیا، در ایران وجود دارد. هدف از انجام مطالعه حاضر، تعیین آنتی‌ژن *ژیاردیا دئودنالیس* در خرگوش‌های خانگی شهرستان اهواز، واقع در جنوب غرب ایران بود. در مجموع، ۵۸ نمونه مدفوع جمع‌آوری شده از خرگوش‌های خانگی، در فاصله زمانی دی ماه ۱۳۹۰ تا آذر ماه ۱۳۹۳، برای تشخیص *ژیاردیا دئودنالیس*، به آزمایشگاه انگل‌شناسی دانشکده دامپزشکی، انتقال داده شده و به کمک دو تکنیک: سانتریفیوژ- فلوتاسیون و استفاده از کیت تجاری آنتی-ژنیکی (شرکت BVT، لیون فرانسه) و به روش ایمونوکروماتوگرافی‌اسی مورد بررسی قرار گرفتند. خرگوش‌های مورد مطالعه به ۲ گروه سنی (کمتر از ۱ سال و بالاتر یا مساوی ۱ سال)، ۴ فصل و بر اساس وضعیت مدفوع، به ۲ دسته (اسهال و بدون اسهال) تقسیم‌بندی شدند. پنج مورد از ۵۸ نمونه مدفوع (۸/۶۲ درصد)، از لحاظ حضور آنتی‌ژن ژیاردیا دئودنالیس و به روش ایمونوکروماتوگرافی‌اسی، مثبت بودند. شیوع عفونت، در خرگوش‌های اسهالی (۴۵/۴۵ درصد؛ ۵ مورد از ۱۱ نمونه)، به شکل معنی-داری بیشتر از خرگوش‌های غیراسهالی (صفر درصد؛ هیچ‌کدام از ۴۷ نمونه) بود ($p = ۰/۰۰۲$). عفونت، شیوع بیشتری در خرگوش-های بالای ۱ سال (۹/۰۹ درصد؛ ۳ مورد از ۳۳ نمونه) در مقایسه با خرگوش‌های جوان کمتر از ۱ سال (۸ درصد؛ ۲ مورد از ۲۵ نمونه) داشت، با این وجود تفاوت معنی‌دار نبود ($p > ۰/۰۵$). شیوع بیماری در خرگوش‌های نر (۹/۵۲ درصد؛ ۲ مورد از ۲۱ نمونه) بیشتر از ماده‌ها (۸/۱۱ درصد؛ ۳ مورد از ۳۷ نمونه) و نیز در فصل پاییز (۱۵/۳۸ درصد؛ ۲ مورد از ۱۳ نمونه) بیشتر بود، اما تفاوت معنی‌داری در ارتباط با جنسیت حیوان و فصل با شیوع عفونت دیده نشد ($p > ۰/۰۵$). آزمایش میکروسکوپی بر روی نمونه‌های مدفوع نشان داد که ۵/۱۷ درصد (۳ مورد از ۵۸ نمونه)، مثبت بودند. برای اطمینان بیشتر، سه نمونه مدفوع و در فواصل ۴۸ ساعت از هر حیوان جمع‌آوری شده بود. نتایج حاضر نشان داد که آنتی‌ژن ژیاردیا، به عنوان یک بیماری مشترک، در خرگوش‌های منطقه اهواز، وجود دارد. اطلاعات بدست آمده نشان داد که تکنیک‌های حساس‌تر نظیر ایمونوکروماتوگرافی‌اسی، جهت تعیین تعداد کم ژیاردیا در نمونه‌های مدفوع ممکن است ضروری بوده و نتایج قابل اعتمادتری داشته باشد.

واژگان کلیدی: *ژیاردیا دئودنالیس*، شیوع، خرگوش، اهواز